BIOSENSOR

The present invention relates to a biosensor, which includes an electrode and a mono-enzyme- or a bi-enzyme-system and uses of

5   the biosensor.

BACKGROUND OF THE INVENTION

Rapid evaluation of food and beverage, such as fish, meat, quality is required in food industry. The biogenic amine con-

10   tent in food has been intensively studied because of their po-
tential toxicity. Histamine is the most biologically active compound from this class, affecting the normal functions of the heart, smooth muscle, motor neurones, and gastric acid secre-
tion. Other biogenic amines, such as putrescine and cadaverine,

15   may amplify the effects caused by histamine intoxication, in-
hibiting the enzymes involved in histamine biodegradation: dia-
mine oxidase and histamine-N-methyl transferase.

Numerous countries adopted maximum levels for histamine in

20   food, especially in fish products. The Italian law has fixed a level of 100 mg/kg food, and similar limits have been adopted by EEC regulations.

Therefore, there is a need for developing of simple and inex-

25   pensive methods for determining of freshness biomarkers. Fresh-
ness biomarkers comprising inositol monophosphate, hypoxanthine and xanthine, these are intermediate degradation products of nucleic acids or biogenic amines, which are produced by micro-
bial decarboxylation of the amino acids, histidine, ornithine,

30   and lysine.

Classical methods for the determination of the content of bio-
genic amines are chromatographic techniques, such as gas chro-
matography, thin layer chromatography, reversed phase liquid

35   chromatography, and liquid chromatography. However, these often require sample pre-treatment and relatively long analysing time, which leads to high costs and make these methods not suitable for routine use.

From US 5,565,329 is a method for determination of histamine
concentration in a sample by determination of the decrease in
dissolved oxygen (DO) known. The method involves adding a solu-
tion of an enzymatic reagent, which have a histamine oxidase
5   activity, into an examination liquid containing the test sample
and detect the sensor output signal. The analyser has a reac-
tion cell provided with a DO electrode. The enzymatic reagent
is a Cu-containing fungal amine oxidase. Which is extracted
from a cellmass belonging to Aspergillus Niger cultured in a
10   culture medium including amine as a nitrogen source. This ap-
proach is not very selective and sensitive.

Enzymatic determination of biogenic amines represents an alter-
native that can solve the above mentioned problems. However,
15   most of the amino oxidase biosensors require a high operating
potential (>500 mV vs. Ag/AgCl), which can lead to high back-
ground currents and low selectivity due to bias signals caused
by electrochemically easily oxidisable interferences, which are
always present in complex matrices, such as food or beverage.
20

SUMMARY OF THE INVENTION
As is clear from the description above a rapid, accurate, sim-
ple and handy analytical instrumental tool is needed for deter-
mination of food hygiene all along the food process line,
25   starting from the source to the consumer.

With the present invention the above mentioned problems have
been solved, the present invention offers a highly sensitive,
selective rapid and very convenient determination and/or detec-
30   tion of the biomarkers in very small amounts.

Thus, the present invention relates to a biosensor for detec-
tion and/or determination of the content of freshness biomark-
ers in food or beverage. The biosensor comprises an electrode
35   and a copper-containing amine oxidase derived from grass pea
(AO, E. C. 1.4.3.6) in a mono-enzyme system, or in a bi-enzyme
system containing said amine oxidase coupled with a peroxidase.

AMENDED SHEET

The mono-enzyme system comprising said copper containing amine oxidase (AO) represents one preffered embodiment of the invention. The amine oxidase may be isolated from grass pea and purified according to Šebela, M., et al, Phytochem. Anal. 1998, 9, 211-222.

Another preferred embodiment of the present invention is the biosensor comprising the bi-enzyme system comprising said copper containing amine oxidase (AO) coupled with a peroxidase (PO) such as horseradish (HRP), soybean, tobacco, sweet potato or palmtree peroxidase.

In another preferred embodiment of the present invention the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium redox polymer. The osmium-based redox polymer is preferably (PVI$_{13}$-dmeOs) of poly- (1-vinyl-imidazole), complexed with [Os- (4,4'-dimetylbipyridine)$_2$ Cl]$^{+/2+}$, and a crosslinking agent such as poly-(ethyleneglycol)-diglycidyl-ether (PEGDGE).

Yet another embodiment of the present invention is the use of the biosensor as an analytical tool in the determination and/or detection of the freshness biomarkers in food.

Other uses and preferred embodiments of the present invention are defined in the use-claims and the subclaims.

DETAILED DESCRIPTION OF THE INVENTION
Amine oxidase represents a class of enzymes with a ubiquitous distribution in mammals, plants and micro-organisms. However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase, fore instance, is a copper-containing amino oxidase, which besides the metal ions also contains an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site.

In methods, where an amine oxidase is used, the enzyme is converting the amine to the corresponding aldehyde, with NH$_3$ and H$_2$O$_2$ release, according to reaction I

AMENDED SHEET

$$R-CH_2-NR_2 + H_2O_2 + O_2 \rightarrow R-CHO + H_2O_2 + NH_3 \qquad I$$
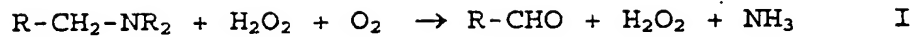
Both oxygen consumption and hydrogen peroxide formation have
been used for monitoring of biogenic amines on the basis of the
above mentioned reaction.

It has surprisingly been shown that the interaction between the
material of the electrode and the enzyme(s) resulted in a very
selective and sensitive biosensor. The electrode has to be of
any electron conducting material, such as noble metals, car-
bon/graphite-based material, conducting salts, conducting poly-
mers etc.

The mono-enzyme based biosensor according to the present inven-
tion is based either on the amine oxidase immobilised on top of
an electrode (DET, direct electron transfer mechanism) or on
amine oxidase crosslinked into a redox hydrogel forming a coat-
ing layer on top of an electrode (MET, mediated electron trans-
fer mechanism).

According to the bi-enzymatic approach of the invention, the
bi-enzyme electrode configuration is based on the enzyme amine
oxidase (AO), from grass pea, and horseradish peroxidase (HRP)
on a solid graphite electrode. The bi-enzymatic system is work-
ing at a potential where biases are minimal. The bi-enzyme
electrodes were prepared either by simply adsorbing the two
enzymes on the electrode surface (DET) or by crosslinking them
into a redox polymer (MET). In the latter case the highly per-
meable and stable redox hydrogel is formed of a poly(1 -
vinylimidazole) complexed with [Os(4,4'-dimetyl-
bipyridine)$_2$Cl]$^{+/2+}$ (PV$_{13}^{T}$-dmeOs) and crosslinked to the enzymes
by a crosslinking agent e.g. poly-(ethylene-glycol)-diglycidyl-
ether (PEGDGE).

The optimal biosensor design was evaluated in terms of sensi-
tivity, selectivity, life- and response-time, and it was used
for the analysis of fish samples stored under different condi-

tions.

In the DET reaction mechanism, the biosensor of the present
invention amine oxidase first converts the amine substrate
5   (e.g. histamine) to an aldehyde product, the active form of the
enzyme being recovered by oxidation of the organic cofactor in
presence of molecular oxygen according to reaction mechanism
II:

10

15   II
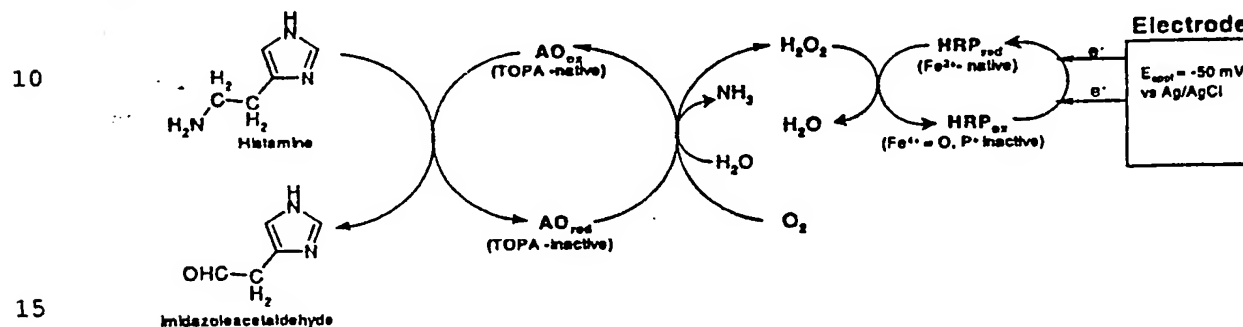
Then the active form of the enzyme being recovered by oxidation
of the organic cofactor in presence of molecular oxygen, see
mechanism II. The hydrogen peroxide formed during the first
20   reaction is subsequently reduced to water by the action of per-
oxidase. The native form of the second enzyme is re-made either
by direct reduction of its heme cofactor on the electrode sur-
face or by receiving electrons from a mediator, maintained in
it's reduced form by the potential applied on the graphite
25   electrode (50 mV vs. Ag/AgCl).

The peroxidase is either reduced by direct reduction of its
heme cofactor (reaction mechanism II) or by receiving electrons
from a mediator (MET), such as an osmium based redox polymer
30   (see reaction mechanism III), maintained in its reduced form by
the potential applied.

35

                                                                     III
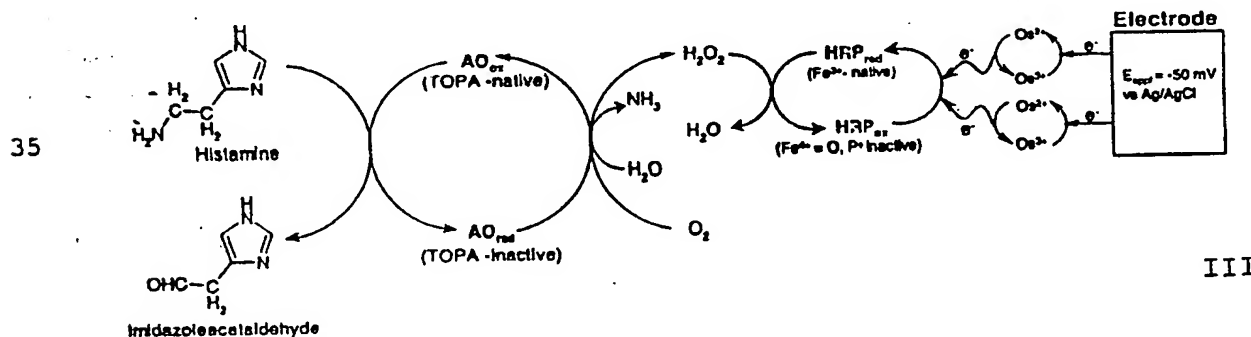
Redox hydro-gels are an effective matrix for enzyme immobilisation resulting in increased stability and the enhanced rates of the electron transfer. The rate of the electron transfer is highly influenced by the composition of the redox hydrogel, as well as by the kinetics of the used enzyme(s). Therefore various biosensor designs were considered in order to find the optimal electrode structure displaying the most efficient rate of electron transfer.

The structure of the redox polymer [Os(4,4'-dimetyl-bi-pyridine)₂Cl complexed to poly(1-vinyl-imidazole)] is shown in following formula:
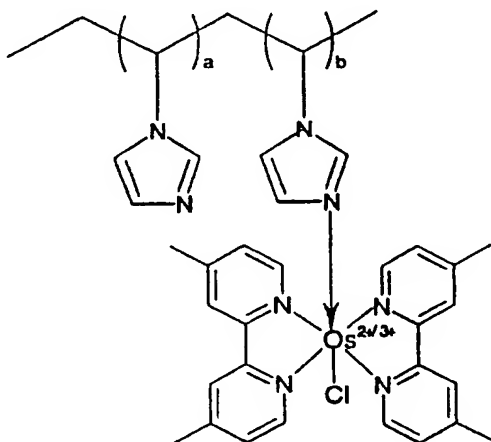
The mono-enzyme- or the bi-enzyme- system is applied on to the electrode in three different ways (type I, II and III). In the following, enzyme means mono-enzyme- or bi-enzyme- system if not otherwise is stated.

Biosensor Type I: the enzyme is applied direct on to the electrode surface (DET). The reaction follows reaction mechanism II.

Biosensor Type II: the enzyme is entrapped in a redox hydrogel and applied on the top of the electrode (MET, one layer electrode). The reaction follows reaction mechanism III.

Biosensor Type III: represent a sequential coating procedure of enzyme and redox polymer (MET, bilayer electrode). The reaction

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follows also reaction mechanism III.

In order to achieve an effective electron transfer all types of
biosensors were optimised with regard to amount of immobilised
5   enzyme and ratio of the used enzyme (Type I), composition of
enzymes : redox polymer : crosslinking agent (Type II) and in-
fluence of electrode coating procedures (Type III).

PREPARATION OF BIOSENSORS
10   The biosensors were prepared by modifying graphite electrodes,
which were prepared as follows:

i)   Rods of spectroscopic graphite (Ringsdorff Werke GmbH,
Bonn, Germany, type RW001, 3.05 mm diameter) were cut, and pol-
15   ished on a wet fine emery paper (Tufback, Durite P1200, Allar,
Sterling Heights, MI).

ii)   The electrode surface was rinsed with water, dried at
room temperature before coating with the enzymes. Three differ-
20   ent electrode types were prepared:

Type I electrodes: were prepared by placing 6 µl of a premixed
solution containing various amounts of AO (stock 20 mg/ml in
phosphate buffer 0.1 M, pH 7.2 (PB)) and HRP (stock 10 mg/ml in
25   PB) on the graphite electrode.

Type II electrodes: were prepared by cross-linking 6 µl of a
mixture formed of AO (stock solution 20 mg/ml in PB), HRP
(stock 10 mg/ml in PB) with an osmium redox hydrogel. The os-
30   mium redox hydrogel consisted of PV$_{13}$-dmeOS (stock 10 mg/ml in
PB) and PEGDGE (5 mg/ml freshly prepared and used within 15
min). The bi-enzyme cross-linked into the redox hydrogel was
placed on the top of the graphite electrode in different ratios
in % by weight (w/w).

35

Type III electrodes: was prepared using a sequential coating
procedure.

Type III a - first a premixed solution 6 μl of HRP_{13}-dmeOs, and
PEGDGE was placed on the top of the electrode. Next, the elec-
trodes were dried for 1 hour before coating with 6 μl of AO
(see table III). .

5

Type III b - first a solution of 6 μl of AO was placed on the
top of the electrode. After drying for 1 hour, the electrodes
were coated with 6 μl of a premixed solution of HRP, PV_{13}-dmeOs,
and PEGDGE.

10

Type III c- in the first step, a drop of HRP solution (6 μl)
was placed an the top of the electrode, and after its drying, a
second layer containing 6 μl of a premixed solution of AO,
PVI13-dmeOs, and PEGDGE was added.

15

Type III d - first a premixed solution of 6 μl of AO, PV_{13}-
dmeOs, and PEGDGE was placed on the top of the electrode. Next,
electrodes were dried for 1 hour before coating 6 μl of HRP.

20   If not otherwise stated, all modified electrodes were stored at
4°C for 14 h in a glass beaker and were rinsed with PB before
use.

The bi-enzyme graphite electrodes were inserted as the working
25   electrode in three electrode cell of wall jet-type placed in a
single channel flow-injection system containing a manual sample
injection valve (Valco Instruments Co. Inc., Houston, TX, USA)
and a 50 μl injection loop.

30   A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to
pump the carrier solution at desired flow rates through Teflon
tubings (0.5 mm i.d.) to the flow cell. A potentionstat (Zäta-
Elektronik, Höör, Sweden) maintained the constant potential
between the working and the reference electrode Ag/AgCl (0.1 M
35   KCl). A platinum wire was used as the counter electrode. The
response current was monitored with a single channel recorder
(Model BD 111, Kipp&Zonen, Delft, The Netherlands).

Operational stability experiments were made using an Automated
Sample Injection Analyser (Ismatec, Glattgurg-Zürich, Switzer-
land) by injecting samples of 100 μM histamine and 50 μM pu-
trescine respectively, with a sample through-put of 30 injec-
5   tions/h using PB as the carrier solution at a flow rate of
0.5ml/min.

The increasing tendency of the apparent Michaelis-Menten con-
stant with the amount of immobilised horseradish peroxidase was
10   attributed to an increase in the thickness of the total protein
loading on the electrode surface. The reducing the analytes
diffusion rate in the film is effected by the influence of the
protein loading. The maximum current, as well as the biosensors
sensitivity trend show that the best combination is the one
15   containing 80% by weight amine oxidase and 20% by weight horse-
radish peroxidase, which has been considered for the further
experiments. The dynamic range for all the studied biosensors
of Type I was 1 - 100 μM for both histamine and putrescine.

20   Different characteristics of Type I biosensors were measured
and calculated for different ratios of amine oxidase AO and
horseradish peroxidase HRP. The values are introduced into ta-
ble I. Where I_{max} and K_m^{app} values are estimated from Michaelis-
Menten equation:

25

$$I = (I_{max} \times [A]) / (K_m^{app} + [A])$$

In table I: A is analyte, S is the sensitivity, calculated as
I_{max}/K_m^{app}, C is the conversion, calculated as $S_{analyte}/S_{H2O2}$ and DL
30   is the detection limit, calculated as 3 S/N (signal- to -noise
ratio).

35

-10-

TABLE I

Type of electrode (w/w)	Analyte	K_m^{app} (µM)	I_{max} (µA)	S (mA/Mcm²)	C (%)	DL (µM)
AO 87% HRP 13%	Histamine Putrescine H_2O_2	279±16 153±15 93±3	1.03±0.02 1.96±0.06 1.80±0.21	50.57±0.82 175.48±1.40 265.13±1.65	19.0 66.2 -	0.16 0.06 -
AO 80% HRP 20%	Histamine Putrescine H_2O_2	332±17 228±15 112±8	134±0.03 3.01±0.07 2.07±0.06	55.28±0.76 180.84±0.95 330.23±1.02	16.6 54.7 -	0.20 0.07 -
AO 67% HRP 33%	Histamine Putrescine H_2O_2	370±22 240±15 153±6	1.30±0.03 3.10±0.01 3.64±0.04	48.13±0.14 176.94±0.87 325.90±0.56	14.7 54.2 -	0.25 0.70 -
AO 50% HRP 50%	Histamine Putrescine H_2O_2	437±43 268±23 175±8	1.22±0.04 3.05±0.10 3.83±0.05	38.24±1.42 155.90±1.26 299.80±0.65	12.7 52.0 -	0.33 0.08 -
AO 40% HRP 60%	Histamine Putrescine H_2O_2	441±23 276±22 206±3	1.16±0.02 3.69±0.06 4.94±0.03	36.03±0.75 183.14±1.11 328.50±0.22	10.9 55.7 -	0.34 0.13 -
AO 33% HRP 67%	Histamine Putrescine H_2O_2	479±41 287±12 211±18	1.37±0.10 3.84±0.06 4.95±0.15	39.18±1.54 183.28±0.61 321.36±1.24	12.2 57.0 -	0.41 0.08 -

Redox hydrogel based biosensors were optimised in order to determine the influence of the redox polycation and the crosslinking agent. Table II shows the obtained results.

If the diffusion barrier increased with the number of added components on the electrode surface, a tendency reflected in the change of the apparent Michaelis-Menten constants. K_M^{app} constant was increased with about 171% for histamine and 125% for putrescine. The introduction of the electrochemical mediator caused a considerable improvement in the bioelectrocatalytic efficiency, as can be seen from increase in the I_{max} with 262%

for histamine and 141% for putrescine and the sensitivity val-
ues with 33% for histamine and 7% for putrescine.

The hydrogen peroxide sensitivity remains practically un-
changed. The detection limit and also the dynamic range for the
studied analytes have also been improved in the case of type II
electrodes.

In the table DR is the dynamic range and all the other symbols
are the same as in Table I.

TABLE II

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)	C %	DL (μM)	DR (μM)
Type I	Histamine	332±17	1.34±0.02	55.29±0.73	16.74	0.16	1-100
	Putrescine	227±16	3.01±0.07	181.64±1.01	55.01	0.06	1-100
	H_2O_2	112±8	2.70±0.06	330.14±1.02			1-100
Type II	Histamine	901±85	4.85±0.41	3.874±1.73	23.07	0.33	1-150
	Putrescine	512±40	7.26±0.53	194.11±1.37	60.73	0.17	1-400
	H_2O_2	977±92	22.8±1.68	319.59±1.63			1-250

The effect of the coating procedure for the type II and type
III biosensors was also studied. Besides coating with a pre-
mixed solutions of all four components, different possibilities
of sequential coatings of the electrode surface, were also
studied, see Table III. Both HRP and AO can be electrically
wired to the redox polymer, and thus cause a partial short-
circuit, when all components are mixed together. This was con-
firmed for the main substrate, putrescine, for which an in-
crease in sensitivity of about 30 % was observed for the two
layer electrodes (type III), compared to the one-layer elec-
trodes (type II).

No considerable change was observed for the other substrate
histamine, the slight decrease in sensitivity being not repre-
sentative considering, the differences of about 10-15 % in

electrode preparation. Clearly, the less sensitive electrode configuration is represented by type III d type electrodes, for which the bias currents due to the wiring of AO are the most explicit. Considering the simplicity of electrode preparation

5 and the small differences in the electrode characteristics between type II and type III electrodes, type II was chosen as optimal electrode design.

TABLE III

10

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)
Type II	Histamine	901±85	4.85±0.41	67.65±1.73
	Putrescine	512±40	7.26±0.53	194.24±1.46
Type IIIa	Histamine	789±35	3.56±0.08	61.80±0.68
	Putrescine	449±34	7.72±0.69	235.53±1.60
Type IIIb	Histamine	687±47	2.66±0.24	53.03±1.55
	Putrescine	473±28	2.04±0.13	59.08±1.19
Type IIIc	Histamine	689±33	2.17±0.06	43.14±0.75
	Putrescine	422±35	7.83±0.82	254.17±1.82
Type IIId	Histamine	649±19	1.90±0.02	40.10±0.42
	Putrescine	425±24	2.14±0.20	68.97±1.49

The influence of various components of the redox hydrogel on the biosensor characteristics is shown in Table IV. The increasing K_m^{app} in the presence of both PVI_{13}-dmeOs and PEGDGE

15 demonstrated that the diffusion of the substrate was limited. This was because of the barrier formed by the mediator and/or cross-linking agent (rigidity of the redox hydrogel) on the surface of the electrode, which also resulted in an increased linear dynamic range. On the other hand, in the presence of

20 crosslinked redox polycationic mediator (PVI_{13}-dmeOs), the I_{max} value was 100% increased suggesting that the final reduction step of the topa cofactor on the electrode surface is the rate-limiting step in the absence of the metyldiator.

In Table IV the response characteristics of different AO bio-
sensors. The AO, PVI$_{13}$-dmeOs and PEGDGE concentrations were 5
mg/ml, 2 mg/ml and 0,5 mg/ml, respectively.

TABLE IV

Type of electrode	K$_m$^{app} (µM)	I$_{max}$ (nA)	S (mA/Mcm^2)	DL (µM)	DR (µM)
AO	375±34	164±6.5	5.99±0.09	2.7	10-100
AO+PEGDGE	755±38	185±5.0	3.35±0.05	4.5	10-150
AO+OVI$_{13}$-dmeOs	770±14	235±2.4	4.18±0.02	3.7	10-150
AO+PVI$_{13}$-dmeOS+ PEGDGE	730±33	360±8.0	6.76±0.05	2.2	10-200

DESCRIPTION OF THE FIGURES

Figure 1:   Shows a voltammogram for 100 µM histamine
             using an AO - HRP modified graphite electrode
             (I).

Figure 2:   Shows the effect of the flow rate on the
             response current and sample throughput of
             Type I biosensors.

Figure 3:   Shows the relative selectivity for different
             amine oxidase substrates, using histamine as
             reference compound, recorded for Type I
             (white) and Type II (black) electrodes.

Figure 4:   Shows the monitoring of freshness in fish
             samples using Type II electrodes. The total
             amine concentration is expressed in histamine
             equivalent units.

The bi-enzyme electrodes were optimised with regard to several
parameters, e.g. working potential, flow rate, influence of
various enzyme ratios and electrode coating procedure.

Hydrodynamic voltammograms were recorded using 100 µM histamine
as substrate and using an AO-HRP-modified type I electrodes in
order to establish the optimal working potential. The voltam-
mogram, together with the ratio between the response and the
5   background current obtained in the same conditions, respec-
tively, are shown in Figure 1. Although the response of the
biosensor drastically increased when the applied potential was
below -100mV so did the background current, which demonstrates
a possible oxygen reduction interference with the biosensing
10   process. A potential of -50 mV vs. Ag/AgCl was chosen as a com-
promise between the response and the background current. The
background current obtained in the same condition (I_0), and the
ratio between them (I/I_0). Conditions: electrode Type I, AO :
HRP 1 : 1 (w/w), flow rate 0,5 ml/min.
15

The influence of carrier flow rate on the biosensor response
for histamine was also considered for type I electrodes, the
results are presented in Figure 2. The decrease in peak height
with the increase in flow rate demonstrates a limitation due
20   either to the bioconversion of the amine substrate by AO or to
the reduction of H_2O_2 by the direct electron transfer between
HRP and the graphite electrode. According to the obtained re-
sults an optimal working flow rate was chosen to be 0.5 ml/min,
as a compromise between the biosensor kinetics and its sample
25   throughput. Conditions: injections of 100 µM histamine, AO :
HRP 1 : 1 (w/w), applied potential -50 mV vs. Ag/AgCl.

Type II biosensors were further characterised with regard to
selectivity, response time, operational and storage stability.
30   Figure 3 shows the relative selectivity for different AO sub-
strates, using histamine as a reference compound, since it is a
biomarker of major interest. As seen, the response for ali-
phatic amines is generally higher than those observed for the
aromatic ones. Also, type II biosensors yielded higher sensi-
35   tivities than type I ones, probably caused by better electron-
transfer kinetics.

The response time of the sensor, calculated as the time elapsed
between 5% and 95% of response height, was fast (less than 1
min).

5   The operational stability of the biosensor was studied both for
histamine and putrescine as substrate. The response current of
the bi-enzymatic enzyme electrode decreased with about 30% and
50% for histamine and putrescine, respectively. This after 10 h
of continuos operation with a sample throughput of 30 injec-
10 tions/h. The storage stability of the electrodes was good, a
decrease of only about 10% and 15% being observed fore hista-
mine and putrescine, respectively, after 10 days of storage.

15 The substrates are histamine His, cystamine Cys, tyramine Tyr,
spermidine Spr, etylenediamine EDA, agmatine Agm, putrescine
Put, cadaverine Cad, Z-Ab-Z-1,4-diamino-2-butene and E-Ab-E-
1,4-diamino-2-butene.

    The optimised biosensor was considered for monitoring bioge-
20 netic amines in real samples. The differentiation between the
signals given by different amines is not possible, only the
total amine content in a sample could be determined. Triplets
of 1.0 g of fish samples were taken from fish-muscles from tro-
bot - *Psetta maxima* - and were kept in different conditions.
25 The samples were homogenised in 10 ml PB. The homogenates were
centrifuged at 13000 g for 60 min at 4°C. The supernatants were
separated and immediately analysed by direct injection into the
flow system. The fish-muscle samples, which had been kept both
at 4°C and 25°C for 10 days, were analysed after extraction in
30 PB by direct injection in the flow system. The total amine con-
tent expressed in histamine equivalents is presented in Figure
4. The maximum accepted limit for total amine concentration in
food products is 100 to 200 mg/kg samples, and a concentration
of 1000 mg/kg is considered to be toxic. After 3 days of stor-
35 age at room temperature, the fish-samples become improper to
consume, while even after 10 days of storage at 4°C there are
not any major changes in the total amine concentration.